IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:
WILLIAMS *et al.*
Application No.: 09/839,946
Filed: April 19, 2001
For: **PEG-Urate Oxidase Conjugates and Use Thereof**

Confirmation No.: 5256
Art Unit: 1652
Examiner: Saidha, T.
Atty. Docket: 2057.0090003/JAG/BJD

Brief on Appeal Under 37 C.F.R. § 41.37

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Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 50-53 was filed on December 20, 2005. In addition, a Pre-Appeal Brief Request for Review was filed on December 20, 2005, a panel decision on which was mailed on January 27, 2006. Appellants hereby file one copy of this Appeal Brief, together with the required fee set forth in 37 C.F.R. § 41.20(b)(2).

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

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I. Real Party In Interest (37 C.F.R. § 41.37(c)(1)(i))

The real parties in interest in this appeal are Mountain View Pharmaceuticals, Inc. having its principal place of business at 3475-S Edison Way, Menlo Park, California 94025 and Duke University, having its principal place of business at Erwin Road, Durham, North Carolina 27710. An assignment assigning all right, title, and interest in and to the patent application from L. David Williams, Mark G.P. Saifer, and Merry R. Sherman to Mountain View Pharmaceuticals, Inc. was recorded in the U.S. Patent & Trademark Office on November 30, 2001 at Reel 012320, Frame 0564. An assignment assigning all right, title, and interest in and to the patent application from Michael S. Hershfield and Susan J. Kelly to Duke University was recorded in the U.S. Patent & Trademark Office on November 30, 2001 at Reel 012320, Frame 0572.

II. Related Appeals and Interferences (37 C.F.R. § 41.37(c)(1)(ii))

To the best knowledge of Appellants, Appellants' legal representative, and Appellants' assignees, there are no other appeals or interferences that will directly affect or be directly affected by, or have a bearing, on a decision by the Board of Patent Appeals and Interferences ("the Board") in the present appeal.

III. Status of Claims (37 C.F.R. § 41.37(c)(1)(iii))

The present application was filed on April 19, 2001 with 41 claims and was assigned U.S. Application No. 09/839,946. In a Preliminary Amendment filed on April 19, 2001, Appellants cancelled claims 1-41 and added new claims 42-76. In a Second Supplemental Preliminary Amendment filed on July 10, 2003, Appellants amended claims 42, 50, 52, 57 and 74.

In an Amendment and Reply Under 37 C.F.R. § 1.111 filed December 11, 2003, Appellants cancelled claims 42-49 and 60-73 and amended claims 50, 53 and 57-59. In an Amendment and Reply Under 37 C.F.R. § 1.111 filed July 6, 2004, Appellants again amended claim 50. Claim 50 was further amended in an Amendment and Reply Under 37 C.F.R. § 1.111 filed May 26, 2005. In an Amendment and Reply Under 37 C.F.R. § 1.116 filed October 20, 2005, Appellants cancelled claims 74-76.

Claims 50-59 are pending in this application, and claims 1-49 and 60-76 have been cancelled.

Claims 50-59 have been rejected.

Claims 50-53 are being appealed.

Claims 54-59 are not being appealed, as the rejection of those claims for obviousness-type double patenting over claims 1-30 of U.S. Patent No. 6,783,965 will be overcome with the filing of a Terminal Disclaimer under 37 C.F.R. 1.321(c).

IV. Status of Amendments (37 C.F.R. § 41.37(c)(1)(iv))

Subsequent to the Final Office Action dated July 20, 2005, claims 74-76 were canceled in Appellants' Amendment and Reply Under § 1.116 filed October 20, 2005.

In an Advisory Action dated December 5, 2005, it was noted that, for purposes of Appeal, the amendments set forth in Appellants' October 20, 2005 reply have been entered.

V. Summary of Claimed Subject Matter (37 C.F.R. § 41.37(c)(1)(v))

The invention as presently claimed is drawn to an isolated tetrameric mammalian uricase, wherein at least about 90% of the uricase is in a tetrameric form and less than about 10% of the uricase is in a non-tetrameric aggregated form. In certain such embodiments, the uricase is porcine liver, bovine liver or ovine liver uricase. In other such embodiments, the uricase is recombinant. In certain other such embodiments, the uricase has the sequence of porcine, ovine or baboon liver uricase.

Support for the sole independent claim involved in the present appeal, claim 50, is found in the specification at least at page 10, lines 15-29; page 16, line 5 through page 17, line 23; and in claims 34, 40 and 41 as originally filed. Claims 51-53 all depend directly or indirectly from claim 50, and add particular features to the uricase recited by claim 50; hence, support for claims 51-53 is found in the same manner as that for claim 50. In addition, claims 51-53 are further supported in the specification at least at page 8, line 30 through page 9, line 10.

VI. Grounds of Rejection to be Reviewed on Appeal (37 C.F.R. § 41.37(c)(1)(vi))

In the final Office Action mailed July 20, 2005 and the Advisory Action mailed December 5, 2005, claims 50-53 were rejected under 35 U.S.C. § 102(b) as being anticipated by Lee *et al.*, *Science 239*: 1288-1291 (1988) (hereinafter "Lee") (Exhibit A).[1]

Accordingly, the sole ground of rejection presented for review on appeal is the rejection of claims 50-53 under 35 U.S.C. § 102(b) as being anticipated by Lee.

[1] Appellants' note that in the final Office Action mailed July 20, 2005, claims 50-59 were also rejected for obviousness-type double patenting over claims 1-30 of U.S. Patent No. 6,783,965 (the '965 patent). As noted in section III above, however, a Terminal Disclaimer under 37 C.F.R. § 1.321(c) over the '965 patent will be filed. Accordingly, the obviousness-type double patenting rejection will be overcome, and the rejection of claims 50-59 on this ground therefore is not presented for review on appeal.

VII. Argument (37 C.F.R. § 41.37(c)(1)(vii))

A. Claims 50-53 Are Not Anticipated By Lee

Claims 50-53 were rejected under 35 U.S.C. § 102(b) as anticipated by Lee. The Examiner relied on Lee as allegedly teaching an isolated tetrameric mammalian uricase, wherein at least about 90% of said uricase is in a tetrameric form and less than about 10% of said uricase is in a non-tetrameric aggregated form. However, Lee does not teach every element recited in claims 50-53. Therefore, the Examiner's rejection of claims 50-53 based on 35 U.S.C. § 102(b) over Lee is legally and factually unfounded.

To establish a *prima facie* case of anticipation under § 102(b), the examiner must show that "each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil of California*, 814 F.2d 628, 631 (Fed. Cir. 1987). *See also Kalman v. Kimberly Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983), *cert denied*, 465 U.S. 1026 (1984). Because the Examiner has failed to establish that each and every element of claims 50-53 is described, either expressly or inherently, in Lee, this rejection of claims 50-53 must be reversed.

B. Despite Examiner's Assertions, Lee Does Not Expressly Disclose Each And Every Element Of Claims 50-53

In making this rejection, the Examiner has contended that Lee discloses the purification of porcine and murine tetrameric uricases that contain at least about 90% tetrameric uricase because the reference mentions that porcine and murine urate oxidase were "purified to homogeneity." *See* Lee at page 1289. The Examiner thus interpreted a "homogeneous" preparation of uricase in Lee to encompass a preparation in which at least about 90% of the uricase is in tetrameric form. *See* Final Office Action mailed July 20, 2005 at pages 3-4. Furthermore, the Examiner pointed to a statement in Lee that

mammalian uricase "exists as a tetramer with an apparent subunit size of 32,000 Daltons" to support his contention that the mammalian uricase disclosed in Lee was 100% in the tetrameric form. *See* Lee at page 1288 and Final Office Action mailed July 20, 2005 at page 3. Appellants respectfully disagree with these contentions, and with this interpretation of Lee upon which these contentions are based, for at least the following reasons.

1. Lee Does Not Expressly Disclose A Tetrameric Uricase As Required By The Present Claims

First, Lee does not *expressly* disclose the purification of *tetrameric* mammalian uricase as recited by the claims of the present application. Lee only indicates that porcine liver and murine urate oxidase were purified to homogeneity. Lee does *not* indicate that at least about 90% of the "purified" uricase was in a tetrameric form. Indeed, Lee does not indicate in *what* form the "purified" uricase was, let alone that at least about 90% of it was in a tetrameric form. Therefore, the Examiner's contentions that Lee discloses an isolated tetrameric mammalian uricase, wherein at least about 90% of said uricase is in a tetrameric form and less than about 10% of said uricase is in a non-tetrameric aggregated form are factually incorrect.

2. Isolation of Tetrameric Uricase, Wherein At Least About 90% Of The Uricase Is In A Tetrameric Form, Was Not Possible Prior To Present Invention

Second, while the Examiner was correct in his assertion that mammalian uricase has a tetrameric structure (as disclosed in the Lee reference), Appellants respectfully point out that this is not the same as isolated uricase being in a non-aggregated, tetrameric form (as claimed in the present invention).[2] As is clearly indicated in the

[2] Additionally, in the Final Office Action, mailed July 20, 2005, on page 6 and the Advisory Action, mailed December 5, 2005, on page 5, the Examiner asserted that Conley *et al.*, *J. Biochem. 187*: 727-732 (1980) (Exhibit B) which discloses uricase from pig liver consisting of four apparently identical subunits "further clarifies that the uricase is tetrameric [four subunits]." However, Appellants again point out that uricase being in a non-aggregated, purely tetrameric form (as in the present invention) is not the same as uricase having a tetrameric structure (as in the Conley reference).

present specification, prior to the present invention, it was not possible to isolate tetrameric uricase wherein at least about 90% of the uricase was in a tetrameric form. Prior to the present invention, isolated preparations of natural and recombinant uricase, including those disclosed in Lee, contained a *mixture* of forms of the enzyme, including high content of non-tetrameric aggregates. *See* specification at page 16, lines 5-16. The estimated percentage of the non-tetrameric aggregated form of the enzyme present in such *purified and isolated* preparations varies from more than 10% to about 80%. *See id.* Indeed, the preparations used in Lee would not contain uricase in which at least about 90% of the uricase was in a tetrameric form and less than about 10% of the uricase was in a non-tetrameric aggregated form. *See* specification at page 16, lines 5-8.

3. Preparations of Uricase Disclosed in Lee Are Not Tetrameric Uricase, Wherein At Least About 90% Of The Uricase Is In A Tetrameric Form

Contrary to the Examiner's contentions, the "homogeneous" uricase preparations of Lee are *not* enriched in the tetrameric form of uricase. Instead the uricase present in these preparations is in the form of monomers, formed from aggregates of isolated uricase by the SDS-PAGE (PolyAcrylamide Gel Electrophoresis in the presence of the detergent, Sodium Dodecyl Sulfate) process used in Lee. The method employed and cited by Lee for assessing the homogeneity of the murine urate oxidase preparations disclosed in that reference *confirms* that Lee is analyzing *monomeric* subunits of uricase rather than the tetrameric form of the enzyme. *See* T.G. Conley and D.G. Priest, "Purification of Uricase from Mammalian Tissue," *Preparative Biochemistry* 9:197-203 (1979) (hereinafter "Conley") (Exhibit C) (cited by Appellants in the Amendment and Reply Under 37 C.F.R. § 1.111 filed November 2, 2004). Conley (and therefore Lee, *citing* Conley at page 1289, 2nd column) used SDS-PAGE to analyze the uricase. While the Examiner was correct that "in a denaturing gel such as SDS/PAGE, only the subunit form [sic] of the uricase is evident," Appellants respectfully contend that the commercial preparation of uricase obtained by Lee for use in SDS-PAGE is not in the "native

tetrameric form" as asserted by the Examiner on page 6 of the Final Office Action mailed July 20, 2005 and on page 5 of the Advisory Action mailed December 5, 2005.

As Appellants noted in their Reply filed in the present matter on December 11, 2003, at pages 26-28, uricase preparations such as those available from Sigma (including Sigma Cat. No. U 3250, the particular commercially available uricase used in the studies in Lee) contain substantial quantities (*i.e.*, more than about 10%) of the non-tetrameric form of the enzyme. Put another way, the Sigma uricase used in Lee does not contain uricase in which at least about 90% of the uricase is in a tetrameric form, as required by the present claims. Moreover, although Lee reports that the Sigma uricase was "purified to homogeneity," Lee at page 1289, col. 2, first full paragraph, this reference does *not* indicate that the "purified" uricase was at least 90% in the tetrameric form. Indeed, the reference does not indicate in *what* form the "purified" uricase was, let alone that at least 90% of it was in the tetrameric form.

This contention is supported by the present specification which discloses that a commercial preparation of uricase, also obtained from Sigma, had to be purified by the methods of the present invention in order to obtain the tetrameric form of uricase. *See* specification at page 20, lines 9-13. The specification further discloses that natural and recombinant uricases isolated from bacteria, fungi, mammals and plants require purification by the methods of the present invention in order to obtain isolated tetrameric uricases. *See* specification at Examples 4-10. In addition, as indicated above, prior to the present invention, uricase was known to rapidly form aggregates larger than tetramers upon being isolated from the tissue. Hence, if the isolated commercial preparation of uricase used by Lee had been analyzed prior to bring denatured during SDS-PAGE analysis, it would have been seen that that uricase preparation did *not* contain at least about 90% of the uricase in a tetrameric form and less than about 10% in a non-tetrameric aggregated form--most of it would instead have been in a non-tetrameric aggregated form. Therefore, the authors of Lee would not be expected to have produced an uricase preparation in which at least about 90% of the uricase was in a tetrameric form; instead, more than 10% of the uricase would have been present in a *non-*

tetrameric aggregated form. This conclusion is further supported by the Declaration Under 37 C.F.R. § 1.132 by Merry R. Sherman, Ph.D. (Exhibit D), and the figures attached thereto, that was filed with Appellants' Amendment and Reply on May 26, 2005.

Given the discussion above, Lee clearly discloses only preparations of uricase in which more than about 10% of the uricase is either: (a) in a non-tetrameric aggregated form (*i.e.*, the commercial preparation); or (b) in a monomeric form after SDS-PAGE analysis. Lee, therefore, does *not* disclose preparations of isolated uricase in which at least about 90% is present in a tetrameric form. Indeed, Lee does not even expressly disclose purifying a tetrameric form of uricase, disclosing only the purification of uricase monomers. Thus, in disclosing "purification to homogeneity" of porcine and murine uricases, Lee is preparing uricase *monomers* and *not* uricase preparations in which at least about 90% of the uricase is tetrameric, as is presently claimed. That is, contrary to the Examiner's contentions, "homogeneity" in Lee does *not* mean "greater than about 90% tetrameric" -- instead, "homogeneity" as used in Lee only means that the uricase has been purified away from non-uricase contaminants. This homogeneous uricase, however, could be present in *any* multimeric form or even in the monomeric form. Given that SDS-PAGE denatures multimeric proteins into their component monomeric forms, a preparation of uricase containing *any* multimeric form of the enzyme -- or even containing a *mixture* of multimeric forms which, as the present specification points out is the most likely form of the commercial uricase used by Lee -- would appear exclusively in the monomeric form after being run on an SDS-PAGE gel. Thus, this statement in Lee relating to homogeneity says nothing about the form, tetrameric or non-tetrameric, in which the uricase of Lee exists prior to SDS-PAGE analysis. Moreover, a homogenous preparation of isolated monomeric uricase -- which is the only isolated uricase expressly disclosed in Lee -- is not the same as an isolated tetrameric uricase which is recited by

the present claims. Thus, as one of ordinary skill would readily appreciate, Lee does not expressly disclose the production of mammalian uricases having the characteristics recited in the present claims.

C. Examiner's Reliance Upon Inherent Anticipation Is Also Factually and Legally Unfounded

If instead, the Examiner was basing this rejection upon the possible inherent disclosure of the claimed uricases in Lee, Appellants respectfully disagree with this approach. To rely on an inherency argument, "the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic *necessarily* flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (PTO Bd. Pat. App. Int. 1990) (emphasis in original). This burden has not been met in the present case, since the Examiner pointed to no disclosure in Lee, nor any sound scientific reasoning, that uricases containing at least about 90% tetrameric uricase "necessarily flow" from the disclosure in Lee. Indeed, as discussed in detail above, the present specification clearly shows that by preparing uricases according to the methods of Lee, one of ordinary skill at best would succeed in preparing uricases that contain *less* than about 90% tetrameric uricase. Indeed, the methods of Lee would result in a uricase preparation in which most, if not all, of the uricase was in a *monomeric*, not tetrameric, form. Thus, any reliance by the Examiner upon inherent anticipation by Lee is factually and legally unfounded.

It is, of course, possible that the intermolecular association of four isolated monomers *in vitro*, under appropriate solution conditions, might theoretically make up a tetrameric uricase. Importantly, however, Lee neither expressly nor inherently discloses such preparations nor the appropriate solution conditions for producing such preparations from the monomeric subunits of uricase obtained in the SDS-PAGE gels disclosed in this reference. As the Federal Circuit has held, a claim can only be anticipated by a

publication if the publication describes the claimed invention with sufficient enabling detail to place the public in possession of the invention. *See In re Donohue*, 766 F.2d 531, 533 (Fed. Cir. 1985); *see also PPG Industries, Inc. v. Guardian Industries Corp.*, 75 F.3d 1558, 1566 (Fed. Cir. 1996) ("To anticipate a claim, a reference must disclose every element of the challenged claim and enable one skilled in the art to make the anticipating subject matter."). Since Lee does not disclose how one of ordinary skill might take the homogeneous isolated monomeric preparations of uricase disclosed in that reference, and produce isolated tetrameric uricase from those monomers, this reference does not enable one of ordinary skill to make the subject matter of the presently claimed invention. Accordingly, for at least these reasons, and under *Donohue* and *PPG Industries*, Lee cannot and does not anticipate the present claims.

D. Lee Does Not Expressly Or Inherently Disclose Each And Every Element Of Claims 50-53

Moreover, under 35 U.S.C. § 102, a claim can be anticipated only if every element in the claim is expressly or inherently disclosed in a single prior art reference. *See Kalman v. Kimberly Clark Corp.*, 713 F.2d 760, 771 (Fed. Cir. 1983), *cert. denied*, 465 U.S. 1026 (1984). The Examiner has pointed to no express disclosure in Lee that would support the Examiner's statement that the "homogeneous preparations of porcine or murine tetrameric uricase comprises the at least about 90% tetrameric form of mammalian uricase claimed." *See* Final Office Action mailed July 20, 2005 at page 4. Furthermore, the present specification clearly shows that by preparing uricases according to the methods of Lee, one of ordinary skill at best would succeed in preparing uricases that contain *less* than about 90% tetrameric uricase. Thus, any reliance upon Lee in an anticipation rejection is factually and legally unfounded.

Accordingly, Lee does not expressly or inherently disclose the presently claimed invention. Hence, under *Kalman*, this reference cannot support a rejection under 35

U.S.C. § 102(b). The rejection of claims 50-53 under 35 U.S.C. § 102(b) over Lee therefore should be reversed by the Board.

VIII. Conclusion

Claims 50-53 are patentable over Lee because the Examiner has failed to establish that Lee anticipates claims 50-53. Therefore, Appellants respectfully request that the honorable Board reverse the Examiner's final rejection of these claims, and remand this application for issue.

Respectfully submitted,

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IX. Claims Appendix

Claim 50. An isolated tetrameric mammalian uricase, wherein at least about 90% of said uricase is in a tetrameric form and less than about 10% of said uricase is in a non-tetrameric aggregated form.

Claim 51. The isolated tetrameric uricase of Claim 50, wherein the uricase is porcine liver, bovine liver or ovine liver uricase.

Claim 52. The isolated tetrameric uricase of Claim 50, wherein the uricase is recombinant.

Claim 53. The isolated tetrameric uricase of Claim 52, wherein the uricase has the sequence of porcine, bovine, ovine or baboon liver uricase.

X. Evidence Appendix

Exhibit A	Lee *et al.*, *Science 239*: 1288-1291 (1988) cited by Appellants in the First Supplemental Information Disclosure Statement filed November 14, 2002 as document AT14 and cited by the Examiner in the Office Action mailed September 11, 2003
Exhibit B	Conley *et al.*, *J. Biochem. 187*: 727-732 (1980) cited by the Examiner in the Office Action mailed July 20, 2005
Exhibit C	T.G. Conley and D.G. Priest, *Preparative Biochemistry* 9:197-203 (1979) cited by Appellants in the Amendment and Reply Under 37 C.F.R. § 1.111 filed November 2, 2004
Exhibit D	Declaration Under 37 C.F.R. § 1.132 by Merry R. Sherman, Ph.D., filed with Appellants' Amendment and Reply Under 37 C.F.R. § 1.111 on May 26, 2005 and acknowledged by Examiner in the Office Action mailed July 20, 2005

XI. Related Proceedings Appendix

To the best of the knowledge of Appellants, Appellants' legal representative, and Appellants' assignees, there are no other appeals or interferences which will directly affect or be directly affected by, or have a bearing on, a decision by the Board of Patent Appeals and Interferences ("the Board") in the present appeal.

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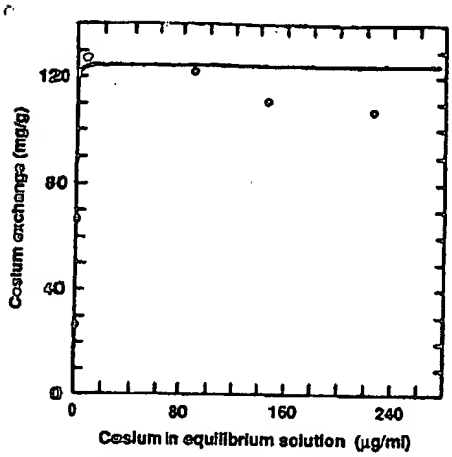

Fig. 2. Cesium exchange isotherm of K-depleted phlogopite mica with constant solid-solution ratio but increasing amounts of cesium.

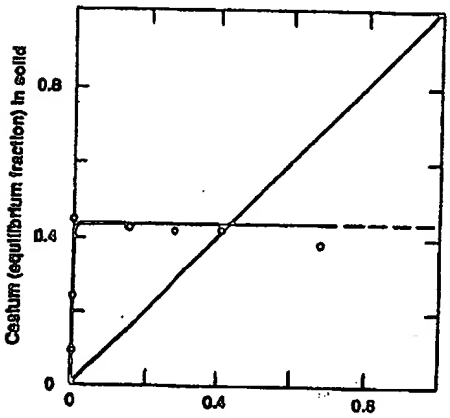

Fig. 3. $Na \rightleftharpoons Cs$ exchange isotherm of K-depleted phlogopite mica with constant amounts of solid, water, and total cations, but variable proportions of Na^+ and Cs^+ cations.

above the diagonal line initially, which shows that Cs^+ is highly preferred over Na^+ initially. However, exchange does not go to completion, as indicated by the cesium exchange capacity of 91.4 meq/100 g, because of the interlayer collapsing and cesium ion trapping effect as described above. Cesium preference over sodium at the initial stages was extremely high, as indicated by the data points of the isotherm falling well above the diagonal line. The extreme preference of cesium over sodium ions in the K-depleted phlogopite mica is further attested by the fact that the total cesium exchange capacity is approximately the same whether sodium is present (91.4 meq of cesium per 100 g) or absent (93.7 meq of cesium per 100 g) in the equilibrating solution. The interlayer spacing of 2.89 Å is ideal for the diffusion of less hydrated cesium ions, just as in the case of γ-zirconium phosphate, which has an interlayer spacing of 2.85 Å (*6*).

A lower interlayer spacing would restrict the diffusion of all ions including cesium, whereas an increase in interlayer spacing would allow much better access to other hydrated ions and thus limit the preference of cesium ions. The ideal interlayer spacing coupled with the high charge density of the layers is instrumental in the selective uptake and trapping of the cesium ions. Partially K-depleted biotite mica has been shown earlier to specifically exchange cesium (*13*), but fully K-depleted phlogopite mica has not been previously studied. Full potassium depletion results in a 12.23 Å *c*-axis *d*(001) spacing that is essential for obtaining the highest capacity and selectivity for cesium exchange.

The selectivity of the K-depleted phlogopite mica for cesium ions in the presence of excess Na^+ and Ca^{2+} ions is compared with some of the cation exchangers that are presently used (Table 1). These results show that K-depleted phlogopite mica is by far the best material for selectively exchanging cesium from concentrated solutions containing Na^+ or Ca^{2+}, these two ions being dominant in natural waters.

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14. The authors acknowledge the financial support of the U.S. Department of Energy through the Division of Materials Science, Office of Basic Energy Sciences, under grant DE-FG02-85ER45204.

28 September 1987; accepted 22 January 1988

Generation of cDNA Probes Directed by Amino Acid Sequence: Cloning of Urate Oxidase

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Urate oxidase (E.C. 1.7.3.3) catalyzes the oxidation of uric acid to allantoin in most mammals except humans and certain primates. The amino-terminal amino acid sequence for porcine urate oxidase was determined and used in a novel procedure for generating complementary DNA (cDNA) probes to this amino acid sequence. The procedure is based on the polymerase chain reaction and utilizes mixed oligonucleotide primers complementary to the reverse translation products of an amino acid sequence. This rapid and simple cDNA cloning procedure is generally applicable and requires only a partial amino acid sequence. A cDNA probe developed by this procedure was used to isolate a full-length porcine urate oxidase cDNA and to demonstrate the presence of homologous genomic sequences in humans.

IN MOST MAMMALS, URATE OXIDASE IS present in the liver, with little or undetectable activity in other tissues. It is associated with the peroxisome and exists as a tetramer with an apparent subunit size of 32,000 daltons (*1*). Humans and certain primates lack this enzyme activity (*2*). Overproduction or elevated serum uric acid levels in man can lead to gouty arthritis. The recent identification of mice with complete hypoxanthine–guanine phosphoribosyl transferase (HPRT) deficiency that do not display any of the symptoms of Lesch-Nyhan's syndrome has raised the possibility that the absence of urate oxidase activity in

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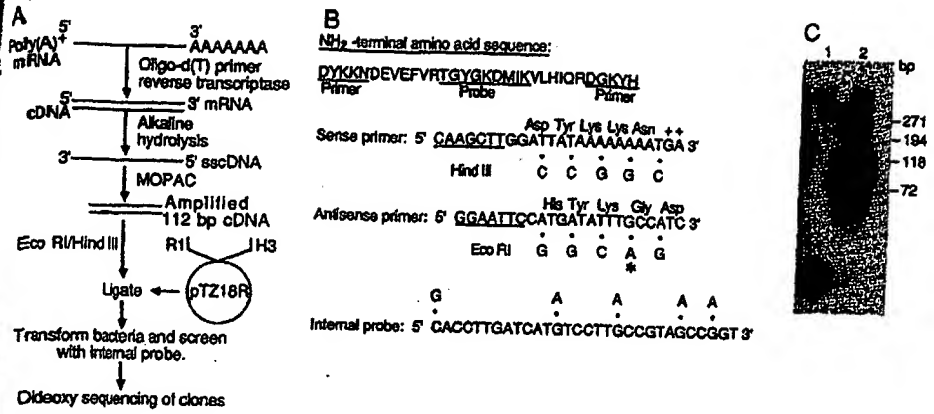

Fig. 1. The strategy for MOPAC cloning, the selection of primers, and probe and product analysis. (**A**) Schematic steps in cloning cDNA based on amino acid sequence and the MOPAC procedure. (**B**) The NH_2-terminal amino acid sequence for porcine urate oxidase and the selection of primers and probe for MOPAC. The sense primer was synthesized to the amino acid sequence 1 to 5. The inclusion of the next two nucleotides from the sixth amino acid is indicated by (++). The antisense primers were synthesized to the amino acid sequence 28 to 32. For both primers, every codon degeneracy was included except for the amino acid glycine, where (*) indicates the selected codon degeneracy. The selection of different restriction enzyme linkers (Eco RI/Hind III) is to facilitate the rescue of amplified cDNA. An internal oligonucleotide probe in the antisense orientation was synthesized for monitoring progress of the MOPAC procedure. (**C**) Autoradiograph of internal probe hybridized to the amplification reaction; lane 1, 10 μl of preamplified reaction mixed; lane 2, 10 μl of postamplified reaction mixed. The annealing and DNA amplification were carried out at 28°C (*18*). The cDNA was separated from primers on a 4% Nusieve agarose gel and transfered by Southern blotting to Zetabind membrane. The probe hybridization was as described (*19*).

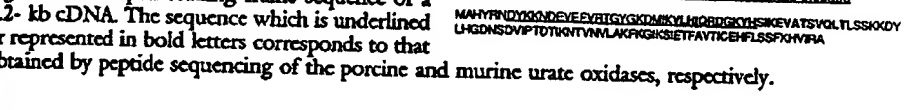

Fig. 2. MOPAC cloned pPUO1 cDNA and the urate oxidase NH_2-terminal amino acid sequence. (**A**) DNA sequence of pPUO1. (**B**) The porcine urate oxidase NH_2-terminal sequence corresponding to the 5′ open reading frame sequence of a 2.2- kb cDNA. The sequence which is underlined or represented in bold letters corresponds to that obtained by peptide sequencing of the porcine and murine urate oxidases, respectively.

the purine metabolism pathway in man may contribute to the development of the neurological symptoms observed in human patients (*3*). For these reasons we have undertaken the molecular cloning of urate oxidase.

A major procedure in complementary DNA (cDNA) cloning involves the synthesis of oligonucleotide probes to a known peptide sequence. However, the degeneracy of the genetic code for all amino acids except methionine and tryptophan requires synthesis of oligonucleotide mixtures for use as hybridization probes (*4–6*). Developing the conditions to distinguish an authentic signal from spurious hybridization is difficult and time consuming with degenerate oligonucleotide probes (*6*). To overcome these limitations, we have developed a procedure, based on the polymerase chain reaction (PCR), for cDNA probe generation from amino acid sequences with highly degenerate codons.

The recently described PCR technique has been used to construct mutations in vitro and to amplify single copy sequences within complex DNA mixtures for facile cloning and analysis (*7*). We now show that specific cDNA probes can be rapidly generated by the PCR when mixed oligonucleotides derived from amino acid sequence are used as primers. The cDNA probe generated by mixed oligonucleotide primed amplification of cDNA (MOPAC) can be used for hybridization studies or for screening a cDNA library for a full-length clone.

Porcine liver urate oxidase was obtained commercially and purified to homogeneity (*8*). Automated Edman degradation of polypeptide (100 picomoles) allowed the determination for the sequence of the first 32 amino acids. Purification of murine urate oxidase to homogeneity was also carried out (*8*) and the amino acid sequence from a cyanogen bromide cleavage peptide was determined. A GeneBank library search (*9*) has revealed no sequence homology to previously cloned genes or peptide sequence.

The strategy used to generate a cDNA probe from this amino acid sequence is described in Fig. 1A. Polyadenylated [poly(A)$^+$] messenger RNA (mRNA) was purified from porcine liver and first strand cDNA was generated with reverse transcriptase from Moloney murine leukemia virus and oligo(dT) primers. After alkaline hydrolysis of the mRNA (*10*), MOPAC was carried out on the single strand cDNA (sscDNA) population. The mixed oligonucleotide primers used for amplification were selected as described in Fig. 1B. The sense and antisense priming regions represented amino acid sequences 1 to 5 and 28 to 32, respectively. The sense primers were synthesized with a Hind III linker including two nucleotides from the codon specifying the sixth amino acid, asparagine, since the addition of these two nucleotides does not alter primer degeneracy. The antisense primers were synthesized with an Eco RI linker: the primers included two out of the four possible codon degeneracies for glycine. For each of these five selected amino acids there are two codon degeneracies. Thus, for each primer mix there are 32 (that is, 2^5) different combinations. An internal oligonucleotide probe was synthesized to represent amino acid sequence 13 to 21 with the most frequently used codons (*11*). The expected size of a successful amplification product would be a cDNA of 112 bp including the restriction enzyme linkers.

After 21 cycles of amplification, the success of MOPAC was determined by Southern hybridization with the internal probe. This probe hybridized strongly to a product of the expected size (112 bp) in the amplified fraction, but not in the preamplified fraction (Fig. 1C). In the preamplified fraction, the signal observed in the region of the gel where the primers migrated suggests a low level of nonspecific hybridization to the primers. These results show that a product of the expected size has been amplified. To rescue this amplified cDNA, the entire amplification reaction was purified by phenol/chloroform extraction, digested with Eco RI and Hind III, and cloned into the Eco RI and Hind III sites of vector pTZ18R.

Seven clones containing the 112-bp insert were identified by in situ hybridization (*10*) of transformed bacterial colonies with the internal probe. One of these (pPUO1) was sequenced by the dideoxy procedure (*12*), which revealed an open reading frame sequence that corresponded to the NH_2-terminal amino acid sequence of porcine urate oxidase (Fig. 2A).

For isolating the full-length porcine urate oxidase cDNA, a porcine liver λ cDNA library was constructed (*13*). About 200,000 primary recombinant phage were screened and about 1 in every 10,000 phage plaques hybridized to the insert from pPUO1 and contained inserts ranging from 1.8 kb to 2.2 kb. Dideoxy sequencing of the 5′ region of a 2.2-kb cDNA gave an open reading frame amino acid sequence that corresponded to the NH_2-terminal sequence for both porcine and murine urate oxidase derived from peptide sequencing (Fig. 2B).

Table 1. Primers used in the synthesis of the pPUO clones. The primers are in the 5′-3′ sense orientation for the seven characterized pPUO clones. The authentic sequence was obtained from the 2.2-kb cDNA, and the underlined nucleotides show the variation from the authentic sequence.

Clone	Sense primer	Antisense primer
pPUO 1	GATTATAAGAAGAATGA	GATGGCAAATATCAC
pPUO 2	GATTATAAGAAGAATGA	GATGGCAAATACCAC
pPUO 3	GATTACAAGAAGAACGA	GACGGCAAATACCAC
pPUO 4	GACTATAAGAAGAATGA	GATGGCAAATACCAC
pPUO 5	GATTATAAGAAGAATGA	GATGGCAAATACCAC
pPUO 6	GACTATAAGAAGAATGA	GACGGCAAATATCAC
pPUO 7	GATTACAAGAAGAATGA	GACGGCAAATATCAC
Authentic	GACTACAAAAAGAATGA	GATGGAAAATATCAC

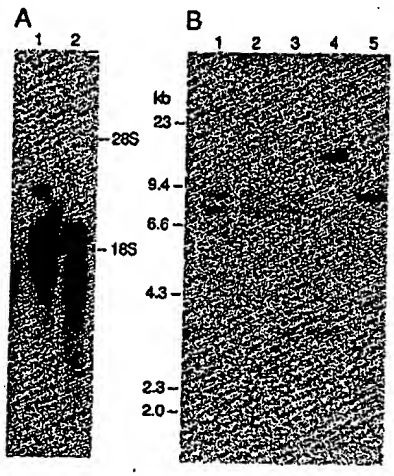

Fig. 3. Northern and genomic Southern analysis for urate oxidase. (**A**) Autoradiography on poly(A)$^+$ mRNA probed with the 2.2-kb cDNA probe: lane 1, 5 μg of murine liver mRNA; lane 2, 5 μg of porcine liver mRNA. (**B**) Autoradiograph of genomic DNA digested with Eco RI and probed with the 2.2-kb cDNA. Lanes 1, 2, and 3 are three unrelated humans; lane 4, mouse; lane 5, hamster. Probe hybridization was carried out at 42°C in the presence of 50% formamide (*19*). The blots were washed in 2× SSC, 0.1% SDS at 65°C.

Thus, the 2.2-kb cDNA is an authentic clone for the porcine urate oxidase.

The pPUO1 sequence is homologous to the 2.2-kb cDNA except for the region corresponding to the primers used for the MOPAC procedure. To determine whether any primers were preferred, six other pPUO clones obtained by the MOPAC procedure were sequenced. The results (Table 1) demonstrate that the generated sequences are identical to the authentic sequence but not the primers' sequence. Thus, the MOPAC procedure will generate authentic sequence even when there are base pair mismatches between the primers and the cDNA. Different primers were found in the pPUO clones although two particular sets of primers were found more frequently than others. There are no obvious rules governing these base pair mismatches, although a C-T mismatch seems more prevalent than a G-A mismatch. The authentic codon usage for glycine, which was not available in the primers, was accommodated by the selection of GGC as the preferred codon in the pPUO clones.

Colony hybridization with a homologous oligonucleotide probe derived from amino acid sequence 6 to 27 indicated that authentic pPUO clones are about 2% of the total transformed bacterial colonies. It is envisaged that with the introduction of a size fractionation step, the frequency for bona fide MOPAC clones would be increased to a level suitable for direct mini-plasmid analysis. Selection of MOPAC clones should be based on size of the expected cDNA product. Several clones having inserts of 70 bp or less were also characterized and found to contain predominantly primers. These appear to be nonspecific amplified products similar to those reported for PCR (*14*).

A Northern blot analysis of the porcine and murine poly(A)$^+$ mRNA with the 2.2-kb cDNA probe identifies mRNAs of about 1.8 and 2.2 kb (Fig. 3A). In the murine sample, a minor 3.0-kb mRNA is also observed. From the size of the mRNA, the 2.2-kb cDNA would represent an apparent full-length cDNA for the porcine urate oxidase. This has been confirmed by the presence of a similar 3′ sequence including a poly(A) tail and a 5′ sequence corresponding to the NH_2-terminal peptide sequence from different full length cDNA clones.

Human and certain primates have no detectable activity for urate oxidase. The total absence of enzyme activity is in keeping with the fact that uric acid is a major excretion product in these mammals (*2*). Surprisingly, a Southern blot analysis of human genomic DNA indicates the presence of restriction fragments that are homologous to porcine urate oxidase cDNA (Fig. 3B). When pPUO1 was used as the probe, a single restriction fragment was observed with human, mouse, and hamster genomic DNA suggestive of a single copy gene. In Old World primates and certain New World primates, a low level of urate oxidase activity is present in the liver and has low in vitro stability compared to the enzyme from other mammals suggesting evolutionary differences (*15*). In humans, the total loss of enzyme activity is probably due to a lack of gene expression since a Northern blot analysis of human liver poly(A)$^+$ mRNA shows an absence of urate oxidase mRNA.

Previous studies have indicated that the presence of imperfectly matched sequences in the same reaction may not interfere with the hybridization of the homologous sequence with its cDNA (*16*). This would suggest the MOPAC procedure will favor the homologous primer as compared to nonhomologous primers. Our results show that this need not be the case. The observation that the characterized pPUO clones do not always share the same primers and that none of these primers are homologous to the authentic sequence indicates that the Klenow fragment of DNA polymerase I (*17*) will efficiently catalyze polymerization using imperfectly matched primers. This is probably a reflection of the flexibility of Klenow polymerase rather than an aberration in DNA hybridization kinetics. This is supported by studies in which PCR has been used to generate mutations in vitro by mispriming (*14*). Mispriming can occur in the MOPAC procedure during the initial annealing or during the subsequent amplification cycles. Our results demonstrate that bona fide probe generation will occur even when there is a 20% base pair mismatch between the primer and the authentic cDNA. The tolerance for such a high level of base pair mismatch is an important advantage of this procedure, since codon degeneracy can vary with different amino acids. The maximum tolerable level of base pair mismatch is not clear although the maximum possible mismatch of 33% was not observed in the clones characterized. We are uncertain of the upper limit for the number of primer combinations although the flexibility in base pair mismatch may reduce the significance of this parameter.

This MOPAC procedure for cDNA cloning has advantages over library screening with degenerate oligonucleotide probes synthesized to the polypeptide sequence. The rapid confirmation of the MOPAC-generated probe by dideoxy sequencing allowed its use for the screening of a cDNA library for full-length clones at maximum hybridization stringency for efficient elimination of unauthentic clones. The MOPAC procedure should have general application to the cloning of genes for proteins whose amino acid sequence is known.

1. C
2. L
3. M
4.
5. F
6. A
7. R
8. P
9. C
10. T
11. F
12. I
13. I
14. E
15. F
16. F
17. F
18. T
19. T
20. C

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19. The internal probe was end-labeled in the presence of [^{32}P]ATP (3000 Ci/mmol) by T4 polynucleotide kinase (*10*). The probe hybridization was carried out at 42°C in 6× SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 0.1% Denhardt's solution, 50 m*M* tris-HCl, *pH* 7.5, and 50 μg/ml denatured herring sperm DNA. The blot was washed in 2× SSC, 0.1% SDS at 42°C.

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Aquatic Productivity and the Evolution of Diadromous Fish Migration

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Diadromous migration, in which some fish species migrate from freshwater and feed in the ocean (anadromous species) and others migrate from the ocean and feed in freshwater (catadromous), has long been perplexing. However, when the distribution of diadromous species is examined with respect to global patterns in aquatic productivity, this apparent paradox is resolved. The contrasting directions of migration can largely be explained by the relative availability of food resources in ocean and freshwater habitats. Oceans are more productive than freshwaters in temperate latitudes, and anadromous species predominate. In contrast, catadromous species generally occur in tropical latitudes where freshwater productivity exceeds that of the ocean.

LARGE-SCALE MOVEMENTS OF ANIMALS are found in many taxonomic groups (*1*). The migrations of diadromous fish species, those that migrate between the ocean and freshwater, are particularly enigmatic because this behavior necessitates physiological changes in osmoregulation (*2*). Diadromous fishes are found in 28 families and include two distinctly different groups: (i) 87 anadromous species, such as salmon (Salmonidae) and lamprey (Petromyzontidae), which are born in freshwater, migrate to the ocean, and return to freshwater to spawn; and (ii) 41 catadromous species, such as some eels (Anguillidae) and mullets (Mugilidae), which are born in the ocean, migrate to freshwater, and return to the ocean to spawn (*3*). The existence of these contrasting directions of migration has long been perplexing. Indeed, it has been described as a paradox in animal migration (*1*). We report that diadromous migrations may occur in fishes because of the differential availability of food resources in ocean and freshwater habitats. Moreover, it is because the relative productivity of oceans and freshwaters is not constant but changes with latitude that the contrasting directions of anadromous and catadromous migration can exist.

In theory (*4*), diadromous life histories will evolve through natural selection only when migration across the ocean-freshwater boundary provides a gain to individual fitness (lifetime reproductive success) that exceeds the costs of this behavior. These costs may include adjustments to physiology, allocation of energy for swimming, and increased probability of mortality during migration. Several authors (*1, 5–9*) have speculated on what factors might favor juvenile fishes deserting their habitat of birth for residency elsewhere. Among these have been decreased predation, decreased disease, decreased physiological stress, or increased food availability. To date, these hypotheses have not been tested quantitatively because of the logistic problems presented by animals that may travel several thousand kilometers and because of our limited knowledge of the life histories of many fish species.

McDowall's (*3*) findings on the global geographic distribution of diadromous species are shown in Fig. 1A. These data indicate latitudinal differences in the worldwide distribution of anadromous and catadromous fishes, with anadromy being more common in temperate (including arctic) latitudes and catadromy in the tropics. Therefore, any hypothesis for the evolution of diadromy must not only provide evidence for a substantial fitness benefit to a diadromous migrant, but must also account for the geographical distribution of diadromy. A hypothesis based on the differential availability of food in the oceans and freshwaters meets these criteria.

Let us first consider whether such a hypothesis can allow for significant fitness benefits through migration. The importance of food intake for body growth (*10*) and the contribution of growth to fitness through decreased mortality (*11*), increased fecundity (*12*), and improved male (*13*) and female (*14*) breeding success have been documented in many fishes. There are also well-studied cases of growth rates increasing with movement across the freshwater-ocean boundary. Juvenile anadromous Pacific salmon, for example, can experience a 10 to 50% increase in their daily growth rate during their first week of ocean life (*11*). In addition, a recent survey by Gross (*4*) of diadromous and nondiadromous populations within seven salmonid species showed that the only significant difference in major life history traits was that individuals in

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Thermodynamics and Stoicheiometry of the Binding of Substrate Analogues to Uricase

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The subunit composition, metal content, substrate-analogue binding and thermal stability of *Aspergillus flavus* uricase were determined. *A. flavus* uricase is a tetramer and contains no copper, iron or any other common prosthetic group. Analytical-gel-filtration and equilibrium-dialysis experiments showed one binding site per subunit for urate analogues. The free energy of xanthine binding was −30.5 kJ (−7.3 kcal)/mol of subunit by equilibrium dialysis and −30.1 kJ (−7.2 kcal)/mol of subunit by microcalorimetry. The enthalpy change for xanthine binding was −15.9 kJ (−3.8 kcal)/mol of subunit when determined from the temperature-dependence of the equilibrium constant and −18.0 kJ (−4.3 kcal)/mol of subunit when measured microcalorimetrically. The thermal inactivation rate of *A. flavus* uricase increases as protein concentration is decreased. This concentration-dependent instability is not due to subunit dissociation.

Uricase (urate–oxygen oxidoreductase, EC 1.7.3.3) from pig liver consists of four apparently identical subunits (Pitts *et al.*, 1974). However, because of the reported metal content of 1 mol of copper/mol of enzyme (Mahler *et al.*, 1955), a single catalytic site has been assumed (Pitts & Priest, 1974). There is now some question about the presence of functional copper in uricase from several other sources (Roush & Shieh, 1963; Nose & Arima, 1968; Itaya *et al.*, 1971; Kosman, 1978).

The results of studies of the binding of substrate analogues obtained with uricase from *Aspergillus flavus* and pig liver have now led to an unambiguous determination of the binding stoicheiometry. Thermodynamic parameters for substrate-analogue binding and thermal-stability properties are also reported.

Materials and Methods

A. flavus uricase was obtained from Boehringer Mannheim Corp. (New York, NY, U.S.A.) as a freeze-dried powder. The enzyme was dissolved in 0.1 M-glycine/NaOH buffer, pH 8.8, and dialysed extensively against this buffer before use. Pig uricase was purified as previously described (Conley & Priest, 1979). Uricase activity was determined by monitoring the decrease in urate absorbance at 290 nm by using a Beckman ACTA C-III recording spectrophotometer with thermostatically controlled cell chamber. Uric acid was obtained from Calbiochem (Los Angeles, CA, U.S.A.), and solutions were prepared fresh daily. Assays were performed at 24°C unless otherwise stated. Assay mixtures consisted of 3.0 ml of 0.1 M-potassium borate buffer, pH 8.8, containing 100 μM-urate at atmospheric O_2 partial pressure. A molar absorption coefficient of $1.23 \times 10^4 M^{-1} \cdot cm^{-1}$ for urate was used (Kalckar, 1947). The concentration of *A. flavus* uricase was determined by using an absorption coefficient ($A_{281}^{1\%}$) of 16.9. The concentration of pig uricase was determined by using an $A_{276}^{1\%}$ value of 11.3 (Mahler, 1963), after this value had been confirmed.

The copper and iron contents of *A. flavus* uricase were determined by complexiметric techniques. Chelating reagents were obtained from Calbiochem. Cuproin (Ochlmann, 1957; Riley & Sinhaseni, 1958), neocuproin (Sherwood & Chapman, 1955; Zall *et al.*, 1957) and bathocuproin (Borchardt & Butler, 1957; Smith & Wilkins, 1953) were used for the detection of copper. *o*-Phenanthroline (Feigl & Caldas, 1957; Peters *et al.*, 1956) and bathophenanthroline (Kingsley & Getchell, 1956; Seven & Peterson, 1958) were used for the detection of iron. Typically, 4 mg of uricase was dialysed against three 4-litre volumes of deionized water, then digested with conc. H_2SO_4. Samples for iron determination were treated with 70% (v/v) $HClO_4$; samples for copper

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determination were treated with 20% (v/v) hydroxylamine. The samples were extracted with isopentanol containing the appropriate chelating agent, and the concentrations of the metal complexes were determined spectrophotometrically.

The molecular weight and the absolute protein concentration of *A. flavus* uricase were determined by sedimentation-equilibrium experiments at 25°C in a Beckman model E analytical ultracentrifuge. The solvent employed was 0.1M-glycine/NaOH buffer, pH 8.8. Experimentally determined values of 1.0024 gm/cm^3 and 1.0203 at 25°C for solvent density and relative viscosity respectively were used. The molecular weight was determined by the short-column meniscus-depletion method of Yphantis (1964). Absolute protein concentration was determined by the method of Babul & Stellwagen (1969).

Gel chromatography was performed on a 2.5 cm × 70 cm jacketed column of Sephadex G-200 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.). The column was equilibrated with 0.1M-glycine/NaOH buffer, pH 8.8, and calibrated by the method of Andrews (1964). Zonal gel filtration (Ackers, 1967; Chiancone *et al.*, 1968) was performed on the same column at 24°C and at 45°C. Typically 20 ml of solution containing from 0.002 to 1.0 mg of protein/ml was pumped on to the column and eluted at a rate of 5 ml/min. The effluent was monitored at 280 nm. Measurement of the number of binding sites by the gel-filtration method of Hummel & Dreyer (1962) was performed with a 2.5 cm × 70 cm jacketed column of Sephadex G-25 at 24°C with saturating concentrations (at least $10^2 \times K_i$) of ligand. Experiments with pig uricase were performed in 0.1M-sodium carbonate buffer, pH 10.2, and those with *A. flavus* uricase in 0.1M-glycine/NaOH buffer, pH 8.8.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate was performed according to the method of Weber & Osborn (1969).

Equilibrium-dialysis experiments were performed with a multichamber equilibrium-dialysis cell (Chemical Rubber Co.). [^{14}C]Xanthine was obtained from ICN (Irvine, CA, U.S.A.) (specific radioactivity 57 mCi/mmol). A 0.5 ml sample of 0.1M-glycine/NaOH buffer, pH 8.8, containing from 0.5 to 2.0 mg of uricase was placed on one side of the dialysis membrane, and 0.5 ml of [^{14}C]xanthine solution was placed on the other. Samples were equilibrated for 18 h at the desired temperature in a shaking water bath. Portions (50 μl) were withdrawn, diluted into 15 ml of Bray's (1960) solution and their radioactivities counted in a Packard Tri-Carb liquid-scintillation spectrometer.

Microcalorimetric experiments were performed with an LKB batch microcalorimeter equipped with gold cells. Experiments were performed at 24°C. Typically, 2 ml of 0.1M-glycine/NaOH buffer, pH 8.8, containing 6 mg of uricase was placed in one side of the reaction cell and 4 ml of xanthine solution was placed in the other. Exact amounts added were determined by weighing the syringes used to transfer the solutions before and after transfer. The cell compartment was sealed and allowed to come to thermal equilibrium. The ligand and enzyme were mixed, and the heat of binding was measured. The calorimeter was calibrated electrically. The heat of dilution of sucrose (Gucker *et al.*, 1939) was measured periodically as a check of calorimeter stability.

Results

Table 1 shows that neither copper nor iron was detected in significant quantities in fully active *A. flavus* uricase. Three different, and relatively specific, chelating agents were used for the detection of copper, and two for the detection of iron. Less than 0.1 mol of either metal/mol of enzyme was detected, and copper or iron, added at a ratio of 0.5 mol of metal/mol of uricase, could be recovered.

Pig liver uricase has been shown to be a tetramer (Pitts *et al.*, 1974). A copper content of 1 mol of copper/mol of uricase (Mahler *et al.*, 1955) has led to the assumption of a single substrate-binding site (Pitts & Priest, 1974). Table 2 shows that the molecular weights, shapes and subunit compositions of *A. flavus* and pig uricases are very similar. A slight difference occurs in the spectral properties of the two enzymes, and there is a pronounced difference in solubility characteristics.

Even if O_2 is nearly completely removed from reaction mixtures by N_2 bubbling, some turnover can still occur, making interpretation of urate-binding data difficult. Therefore the unreactive urate analogues xanthine and trichloropurine were used to

Table 1. *Absence of copper and iron from A. flavus uricase*

Acid digests of the proteins were extracted with isopentanol containing the chelating agents cuproin, neocuproin and bathocuproin for copper, and *o*-phenanthroline and bathophenanthroline for iron. Concentrations of the metal complexes were determined spectrophotometrically. Samples (1 ml) containing 4.0 mg of uricase (32 μM) were used in each experiment. Values shown reflect the number of mol of metal bound/mol of enzyme.

Metal	Added metal (mol/mol of uricase)	Recovered metal (mol/mol of uricase)
Copper	None	$\leqslant$0.06
Copper	0.50	0.46 ± 0.05
Iron	None	$\leqslant$0.08
Iron	0.50	0.58 ± 0.03

Table 2. *Physical properties of pig and A. flavus uricases*
The specific activity of the *A. flavus* uricase was 16.9 units (μmol/min)/mg.

Property	Pig uricase	*A. flavus* uricase
Molecular weight	125000*	130000§
$s^0_{20,w}$ (S)	6.85*	6.75‖
$f/f_{min.}$	1.34*	1.29§‖
Subunit molecular weight	32000*	32000¶
$\lambda_{max.}$ (nm)	276†	281
$A^{1\%}_{\lambda max.}$	11.3†	16.9**
Solubility (mg/ml)		
pH 10.0‡	<5	>10
pH 7.0‡	<0.05	>10

* Values are those reported by Pitts *et al.* (1974).
† Values are those reported by Mahler (1963).
‡ Buffers used were 0.1 M-sodium carbonate at pH 10.0 and 0.1 M-potassium phosphate at pH 7.0. The enzymes were dissolved by vortex-mixing in the appropriate buffer for 2 min, centrifuged to remove undissolved protein, and the dissolved protein concentration was determined by measuring the absorbance at the appropriate $\lambda_{max.}$.
§ Determined from sedimentation-equilibrium data.
‖ Determined from sedimentation-velocity data.
¶ Determined from relative mobility on polyacryamide-gel electrophoresis in the presence of sodium dodecyl sulphate.
** The absolute protein concentration was calculated by the method of Babul & Stellwagen (1969).

investigate the number of purine-binding sites on uricase. Both of these compounds behave as classical competitive inhibitors of uricase with respect to urate (xanthine, $K_i = 3.0\,\mu M$; trichloropurine, $K_i = 0.4\,\mu M$). Gel-filtration experiments, performed by the method of Hummel & Dreyer (1962), were used to estimate an *A. flavus* uricase binding stoicheiometry of 3.8 ± 0.4 for xanthine and 4.1 ± 0.3 for trichloropurine. The relative insolubility of pig uricase prevented precise stoicheiometry estimates, but both trichloropurine and xanthine bound at a ratio significantly greater than 1:1.

Fig. 1 shows that inactivation occurred when *A. flavus* uricase samples were maintained at elevated temperatures. Similar inactivation profiles obtained with pig uricase have been shown to fit a reversible first-order two-state (fully active and totally inactive enzyme) kinetic model (Pitts *et al.*, 1974). Such a model can also be used for *A. flavus* uricase. Fig. 2 shows that the thermal inactivation of *A. flavus* uricase is also enzyme-concentration-dependent. Rate constants for both the forward and the reverse processes are shown in Table 3. The insolubility of pig uricase made such studies difficult with that enzyme.

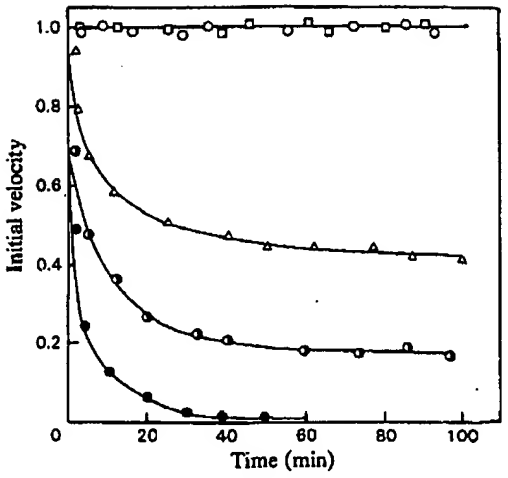

Fig. 1. *Decrease in A. flavus uricase initial velocity with time at elevated temperatures*
Samples (1 ml) containing 0.010 mg of uricase/ml in 0.1 M-glycine/NaOH buffer, pH 8.8, were immersed in a water bath at 24°C (○), 35°C (□), 45°C (△), 51°C (◑) or 63°C (●). After 1.5 min, for temperature equilibration, samples were withdrawn and initial rates were determined at 290 nm in 0.1 M-sodium borate buffer, pH 8.8, containing 100 μM-urate at 24°C. A value of 1 on the relative initial-velocity scale corresponds to a specific activity of 16.9 units/mg.

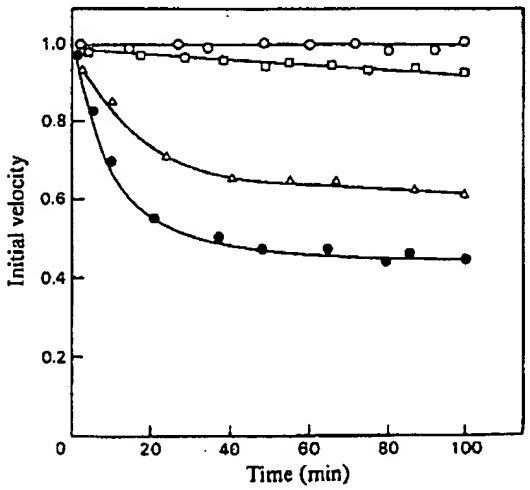

Fig. 2. *Enzyme-concentration-dependence of the decrease in A. flavus uricase initial velocity with time at 45°C*
Concentrations used were 0.91 mg/ml (○), 0.106 mg/ml (□), 0.053 mg/ml (△) and 0.010 mg/ml (●). Other conditions are the same as in Fig. 1.

Fig. 3 shows that subunit dissociation is not the source of the concentration-dependent thermal instability of uricase. Zonal analytical-gel-filtration experiments were conducted over a protein con-

Table 3. *Apparent first-order rate constants for thermal inactivation of A. flavus uricase*

Rate constants were calculated from the results shown in Figs. 1 and 2 by the method previously reported (Pitts *et al.*, 1974).

Temperature (°C)	Uricase (mg/ml)	k_f (min^{-1})	k_b (min^{-1})
45	0.053	0.055	0.096
45	0.010	0.084	0.067
51	0.010	0.077	0.017

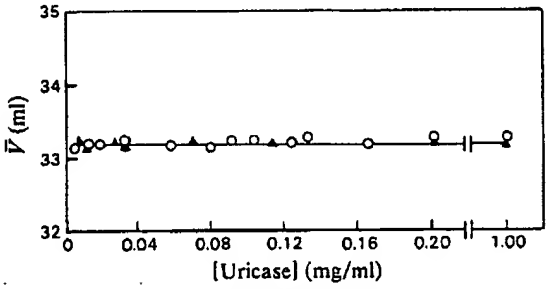

Fig. 3. *Zonal gel filtration of A. flavus uricase*

A 2.5 cm × 70 cm column of Sephadex G-200, equilibrated with 0.1 M-glycine/NaOH buffer, pH 8.8, was used at 24°C (○) and at 45°C (▲). Typically 20 ml of protein solution was pumped on to the column and eluted at a flow rate of 5 ml/h. The elution volume ($\bar{V}$) is defined as the centroid of the leading edge of the protein zone (Ackers, 1967).

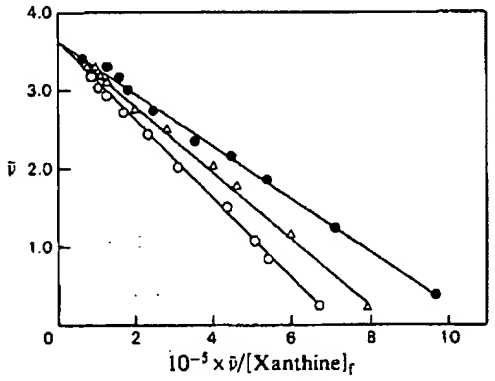

Fig. 4. *Equilibrium-dialysis measurements of xanthine binding to A. flavus uricase*

A sample (2 mg) of uricase in 0.5 ml of 0.1 M-glycine/NaOH buffer, pH 8.8, was placed on one side of a membrane, and xanthine in the same buffer system was placed on the other. After 18 h the concentration of [^{14}C]xanthine was determined on each side of the membrane, and the amount bound was calculated. Results obtained at 16°C (●), 24°C (△) and at 35°C (○) are plotted in accordance with the Scatchard (1949) relationship.

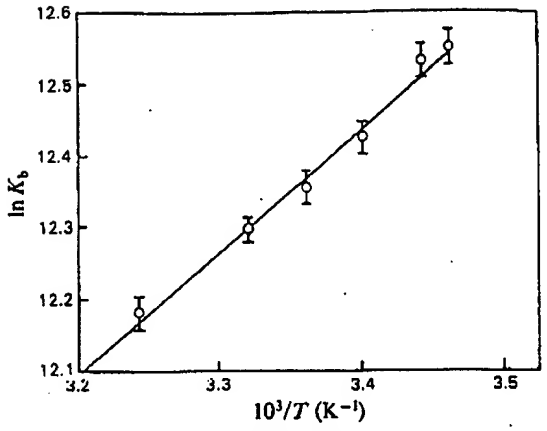

Fig. 5. *Temperature-dependence of the A. flavus uricase–xanthine binding constant*

Conditions were the same as described in Fig. 4. Points represent the averages of at least five separate determinations and error bars the standard deviation.

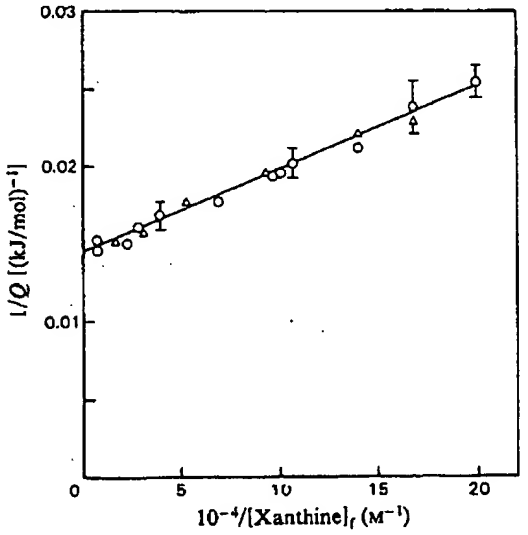

Fig. 6. *Reciprocal plot of microcalorimetrically determined heat associated with xanthine binding to A. flavus uricase*

The heat of dilution of the xanthine solution was blanked out in each experiment; the heat of dilution of the enzyme was measured separately and the binding heats were corrected accordingly. As recommended by Bolen *et al.* (1971), $Q_{max.}$ was measured at high ligand concentration and used to calculate the concentration of free xanthine. Binding was performed at atmospheric O_2 partial pressure (○) and at 100% N_2 (△).

centration range that exhibited inactivation. No change was observed in the elution volume ($\bar{V}$) of the leading edge of the protein band. Results were identical at 24°C and 45°C.

Table 4. *Thermodynamic parameters for the binding of xanthine to A. flavus uricase at 24°C*

Values of ΔG^0, ΔH^0 and ΔS^0 are given per mol of subunit. Free-energy change was calculated from the relationship $\Delta G^0 = -RT \cdot \ln K_b$, and entropy change was calculated from the relationship $\Delta G^0 = \Delta H^0 - T\Delta S^0$.

	K_b (M^{-1})	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 ($J \cdot mol^{-1} \cdot K^{-1}$)
Equilibrium dialysis	2.33×10^5	−30.5	−15.9	49.4
Microcalorimetry	2.11×10^5	−30.1	−18.0	41.0

Equilibrium dialysis was used to measure xanthine binding. Linear Scatchard (1949) plots were obtained at each temperature used (see Fig. 4). A binding constant of $2.33 \times 10^5 M^{-1}$ was estimated at 24°C. Identical results were obtained over the enzyme concentration range 0.5–2.0 mg/ml.

Binding constants were determined as a function of temperature over the range 16–35°C. When they were plotted according to the van't Hoff relationship (see Fig. 5), an enthalpy change of −15.9 kJ (−3.8 kcal)/mol of binding sites was obtained. Microcalorimetry was also used to measure the enthalpy change for xanthine binding at 24°C (see Fig. 6). Extrapolation to infinite xanthine concentration allowed an estimate of −18.0 kJ (−4.3 kcal)/mol of binding sites. Essentially identical results were obtained in 0.1 M-glycine and 0.1 M-Tris buffer systems at pH 8.8. Since these two buffers have different heats of ionization (Hinz *et al.*, 1971), proton dissociation does not appear to accompany xanthine binding. A summary of the thermodynamic parameters for xanthine binding, including the entropy change at 24°C, is shown in Table 4.

Discussion

Uricase from *A. flavus* and pig liver are both tetrameric. The *A. flavus* enzyme contains one purine-binding site per subunit. The relative insolubility of the pig enzyme made stoicheiometry estimates less precise. Nevertheless urate analogues bound at a ratio clearly greater than 1 molecule per tetramer. Therefore previous assumptions of substrate-binding stoicheiometry, based on apparent copper content (Pitts & Priest, 1974), are incorrect. Since copper is not present in *A. flavus* uricase, and if present in the pig enzyme is not required for purine binding, it is unlikely that copper plays a direct role in enzymic catalysis of urate oxidation.

Both the *A. flavus* and pig enzymes are thermally inactivated in a manner that fits a reversible first-order kinetic model. The *A. flavus* enzyme also exhibits a protein-concentration-dependence for this thermal inactivation. Subunit dissociation was ruled out as the cause of the concentration-dependent lability of the *A. flavus* enzyme. Dissociation of a prosthetic group could account for the concentration-dependence, but the present evidence suggests that the enzyme does not contain a readily dissociable cofactor.

Below 35°C the enzyme was sufficiently stable for us to conduct equilibrium binding studies with urate analogues. Close inspection of binding isotherms can be used to test for interaction between binding sites. With the use of linearized plotting forms no evidence could be obtained for such interactions by equilibrium dialysis or microcalorimetric measurements. Both techniques yielded essentially the same binding constant at 24°C. This constant compares quite well with the steady-state kinetic inhibition constant, K_i (3.0 μM), for xanthine. For comparison, the binding constant in Table 4 can be expressed as a dissociation constant K_d (4.3 μM).

The enthalpy change for xanthine binding to *A. flavus* uricase, as determined from the temperature-dependence of the binding constant and by microcalorimetry, are in very close agreement. No protein dissociation could be detected from the microcalorimetric measurements, nor would such close agreement be expected between the two techniques if such were the case. The relatively small enthalpy change when combined with the equilibrium constant at 24°C yields a positive entropy change. Neither the enthalpy term nor the entropy term dominate the free-energy equation at 24°C. Such an entropy change is consistent with a decrease in the degree of hydration on binding of xanthine.

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PURIFICATION OF URICASE FROM MAMMALIAN TISSUE

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ABSTRACT

A simple, rapid procedure for the purification of uricase from mammalian tissue is reported. The procedure is based on the precipitation of mammalian uricase under certain dialysis conditions, and on its low solubility near neutral pH. Exceptionally high yields of homogeneous enzyme are obtained.

INTRODUCTION

The starting material for purification of porcine liver uricase has typically been dried powders prepared from acetone precipitates of liver extracts[1-5]. Much of the original uricase activity in liver is lost during this procedure, ultimately resulting in relatively low yields. Affinity chromatographic techniques for purification have been developed by Batista-Viera et al.[6]. Partially purified commercial preparations are used as starting material. The procedure reported here allows unusually high recovery of homogenous uricase using frozen liver as the

starting material. The procedure requires only two steps, with an unusual dialysis mediated precipitation[3,4] responsible for the major purification. This procedure is suitable for large scale preparations of homogeneous uricase.

MATERIALS AND METHODS

Porcine liver was routinely obtained from local slaughterhouses and frozen at -20° until used. Liver frozen for as long as a year showed no loss in uricase activity.

Uric acid was obtained from Calbiochem. Uricase activity was determined by measuring the rate of decrease in absorbance at 290 nm, with a Beckman Acta C-III recording spectrophotometer. A molar extinction coefficient for uric acid of 12,300 was used[7]. Standard assay conditions were 100 μM urate in 0.1 M sodium borate buffer, pH 8.8, at 25°[8]. The concentration of protein in crude preparations was determined by the method of Warburg and Christian[9]. The concentration of purified uricase was determined from the extinction coefficient $E^{1\%}_{276} = 11.3$[10].

Polyacrylamide gel electrophoresis experiments in the presence of sodium dodecyl sulfate were conducted according to the procedure of Weber and Osborn[11]. Protein bands were typically stained with Coomosie Brilliant Blue R (Sigma Chemical Co.).

RESULTS

Mahler et al. reported that porcine uricase would precipitate upon dialysis against tris buffer, and incorporated this finding into a procedure to purify the enzyme [3,4]. This tendency of mammalian uricase to precipitate upon dialysis is not restricted

to a particular buffer system. Table I shows uricase activity remaining in solution after the enzyme has been dialyzed against various buffers. The pH of the buffers against which the enzyme was dialyzed did not change significantly during the course of any experiment. Essentially all of the activity lost from the supernatant could be accounted for by redissolving the precipitated protein in 0.1 M sodium carbonate, pH 10.2, with vigorous vortexing. Table I further shows that 80% of the porcine uricase had precipituted when dialyzed for 48 hours against the same buffer in which it is normally stored (0.1 M sodium carbonate, pH 10.2). No precipitation is noted when the enzyme is stored without dialysis. Dialysis against 0.01 M tris, pH 8.0, caused essential-

TABLE I

Uricase Activity Remaining After Dialysis[a]

Dialysis Medium	Percentage Activity Remaining Dialysis Time (hr) 6	12	24	48
0.1 M Sodium Carbonate, pH 10.2	92	69	32	18
0.1 M Glycine, pH 8.5	88	38	8	2
0.01 M Glycine, pH 8.5	86	40	6	<1
0.1 M Tris, pH 8.0	42	16	4	<1
0.01 M Tris, pH 8.0	46	6	2	<1
1.0 M Sodium Chloride, pH 7.2	85	73	42	22

[a]One ml samples containing 0.5 mg per ml of porcine uricase (sp. act. 8.1) in 0.1 M sodium carbonate buffer, pH 10.2, were dialyzed against 1.8 L of each buffer. At the end of the appropriate time period a sample was removed from the dialysis medium, centrifuged to remove precipitated enzyme, and the supernatant assayed for uricase activity.

ly all of the uricase activity to precipitate within 12 hours. Therefore this buffer system was chosen for incorporation into the following purification procedure.

Porcine liver (from 50 to 600 gms) was homogenized in two volumes of distilled water in a blender. The suspension was centrifuged in a Sorvall RC-2B centrifuge at 15,000 g for 15 minutes. The pelleted material was washed with distilled water by blending and recentrifuged. Uricase was solubilized by placing the pellet in 6 volumes of 0.1 M sodium carbonate buffer, pH 10.2 (based on the original amount of tissue used) and blended at high speed for 2 minutes. After centrifugation, the pH of the supernatant was lowered to 6.8 with 3 M acetic acid. The flocculant precipitate was removed by centrifugation at 20,000 g. Uricase precipitated upon adjustment of the pH to 4.7 with glacial acetic acid. The precipitated material was redissolved in a minimal amount of 0.1 M sodium carbonate, pH 10.2, with vigorous agitation, and dialyzed against 0.01 M tris, pH 8.0, for 12 hours to precipitate the enzyme. After washing with 0.05 M glycine, pH 8.5, the enzyme was dissolved by vortexing in 0.1 M sodium carbonate, pH 10.2. The dialysis/precipitation step and glycine wash was repeated once. Further reprecipitation caused a decrease in specific activity. The purified enzyme was stored in 0.1 M sodium carbonate, pH 10.2, at 4°. Under these conditions the enzyme remained in solution, with a loss in specific activity of less than 10% after six months.

Table II shows the results of a typical purification. Twenty mg of uricase per kg of porcine liver, with a specific activity of approximately 8.5, was obtained routinely. The enzyme is homogeneous upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. A slower moving, minor band, previously observed[12] upon electrophoresis of commercial preparations of porcine liver uricase was not present. It could be generated by treatment of our preparations with glycerol, a component of the media in which the commercial preparations were stored. The catalytic properties of uricase prepared by the procedure presented here are identical to those of uricase commercially prepared[12].

TABLE II

Purification of Porcine Uricase[a]

Purification Step	Volume (ml)	Total Protein (mg)	Total Activity	Specific Activity	Yield (%)
pH 10.2 Carbonate Extract	1210	19,118	83.5	0.004	100
pH 6.8 Supernatant	1185	15,168	72.9	0.005	94.9
pH 4.7 Precipitate, Redissolved in pH 10.2 Carbonate	100	3,330	55.7	0.017	66.7
Enzyme Precipitated by Dialysis *vs* Tris, Redissolved in pH 10.2 Carbonate	10	6.9	43.2	6.22	51.7
Enzyme Reprecipitated by Dialysis *vs* Tris, Redissolved in pH 10.2 Carbonate	10	4.3	36.0	8.38	43.1

[a]Starting material was 212 gm of frozen porcine liver.

DISCUSSION

Essentially no uricase activity is solubilized when mammalian liver is homogenized in water at neutral pH. As the pH is increased, uricase becomes more soluble. This property provides a convenient means for extraction. Water soluble protein can be removed by washing with water at pH 7.5, allowing the subsequent extraction of uricase at pH 10.2. After extraction, reprecipitation by lowering the pH provides a further increase in specific activity and concentration of the enzyme.

Mammalian uricase precipitates upon dialysis, even though the reservoir buffer is essentially the same as the buffer in which the enzyme is dissolved. Precipitation does not occur upon standing. A nucleation process appears to require the dialysis conditions. A marked purification of the enzyme can be obtained by this very selective precipitation. The enzyme prepared by precipitation in this manner is homogeneous by electrophoretic criteria and behaves otherwise in a manner identical to uricase prepared by other techniques.

The high yield of homogeneous uricase that can be obtained in large quantities by this simple purification approach has obvious advantages over more classical techniques. Even a recently developed affinity chromatographic approach[6], while able to yield highly homogeneous uricase, nevertheless is limited by the chromatographic steps and cannot give rise to the quantities of uricase yielded by the present technique. This method should prove useful for pilot plant or production scale quantities of uricase from mammalian sources.

ACKNOWLEDGMENTS

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For: **PEG-Urate Oxidase Conjugates and Use Thereof**	

Declaration of Merry R. Sherman Under 37 C.F.R. § 1.132

Commissioner for Patents
PO Box 1450
Alexandria, VA 22313-1450

Sir:

I, the undersigned, **Merry R. Sherman,** declare and state that:

1. I am a co-inventor of the above-captioned U.S. patent application number 09/839,946, filed April 19, 2001, entitled, "PEG-Urate Oxidase Conjugates and Use Thereof."

2. I am also the President of Mountain View Pharmaceuticals, Inc. ("MVP"), a co-assignee of the present application by virtue of an assignment from L. David Williams, Mark G. P. Saifer and Merry R. Sherman to MVP executed on September 29, 1999, and recorded in the U.S. Patent and Trademark Office on November 30, 2001, beginning at Reel No. 012320, Frame No. 0564.

3. My *curriculum vitae* is attached as **Exhibit A**.

4. I have reviewed the above-identified patent application and the Office Action dated January 26, 2005. I would like to address certain remarks raised by Examiner Saidha in the Office Action.

5. On page 2 of the Office Action, Examiner Saidha states that Lee *et al.* (hereinafter "Lee") discloses that mammalian uricase is a "tetramer with subunit size of 32,000 daltons." The Examiner uses this statement in Lee to support his assertion that the mammalian uricase in Lee was 100% in the tetrameric form. However, the mammalian uricase referred to by Lee, in the sentence pointed out by the Examiner, refers to mammalian uricase "associated with the peroxisome." The mammalian uricase "associated with the peroxisome" is very different from the purified mammalian uricase disclosed by Lee. Specifically, while mammalian uricases *in vivo* (*i.e.*, associated with the peroxisome) exist as a tetramer, isolated purified preparations of natural and recombinant uricase, as indicated in the present specification and as disclosed by Lee, usually contain a mixture of aggregated non-tetrameric forms of the enzyme, in addition to the tetrameric form. *See* specification at page 16, lines 5-8.

6. As explained in the present specification, a mixture of various aggregated forms of the uricase, other than the tetrameric form, is believed to be highly immunogenic. *See* specification at page 16, lines 8-16. However, the present application teaches a method for isolating a tetrameric form of uricase from a solution containing natural and/or recombinant forms of uricase, thereby reducing the immunogenicity of the uricase without disrupting its activity. *See* specification at page 10, lines 15-29. The purification procedure, as outlined in the present specification, results in the chromatographic results shown in attached Figures 1 and 2. Figures 1 and 2, attached hereto, were disclosed in U.S Patent No. 6,783,965 ("the '965 patent") as Figures 2 and 3. MVP is the assignee of the '965 patent.

7. Figure 1 illustrates size exclusion HPLC analysis on a Pharmacia Superdex 200 column (1x30 cm) of the load and selected fractions from a preparative Mono Q chromatography of porcine uricase containing the mutations R291K and T301S (PKS uricase) showing data obtained by a light scattering detector at 90°C (upper curves) and by absorbance at 276 nm (lower curves). Figure 2 illustrates size-exclusion analyses of fractions from a Mono Q column, showing data obtained by a light scattering detector at 90° and by absorbance at 276 nm, as in Figure 1.

8. The top panel in each of Figures 1 and 2 illustrates that octamers and larger non-tetrameric aggregates account for greater than 10% of the uricase present in isolated natural and recombinant uricase preparations, such as those disclosed in Lee. After the purification procedure of the present application is performed, the majority (*i.e.*, at least about 90%) of the uricase present is in a tetrameric form. *See* bottom panel in each of Figures 1 and 2. Thus, these data clearly demonstrate that the purification procedures disclosed in the present application are required in order to obtain the presently claimed isolated mammalian uricases in which at least about 90% of the uricase is in the tetrameric form. Accordingly, without specifically purifying their uricase preparations according to the methods of the present application, the authors of Lee would not be expected to have produced an uricase preparation in which at least about 90% of the uricase is in a tetrameric form.

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements

and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the present patent application or any patent issued thereon.

Respectfully submitted,

Merry R. Sherman, Ph.D.

Date: May 25, 2005

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Figure 1
U.S. Appl.No 09/839,946

Size-Exclusion HPLC on Superdex 200 of Unfractionated PKS Uricase (Load) and Mono.Q Column Fractions in the Low-Salt Pool

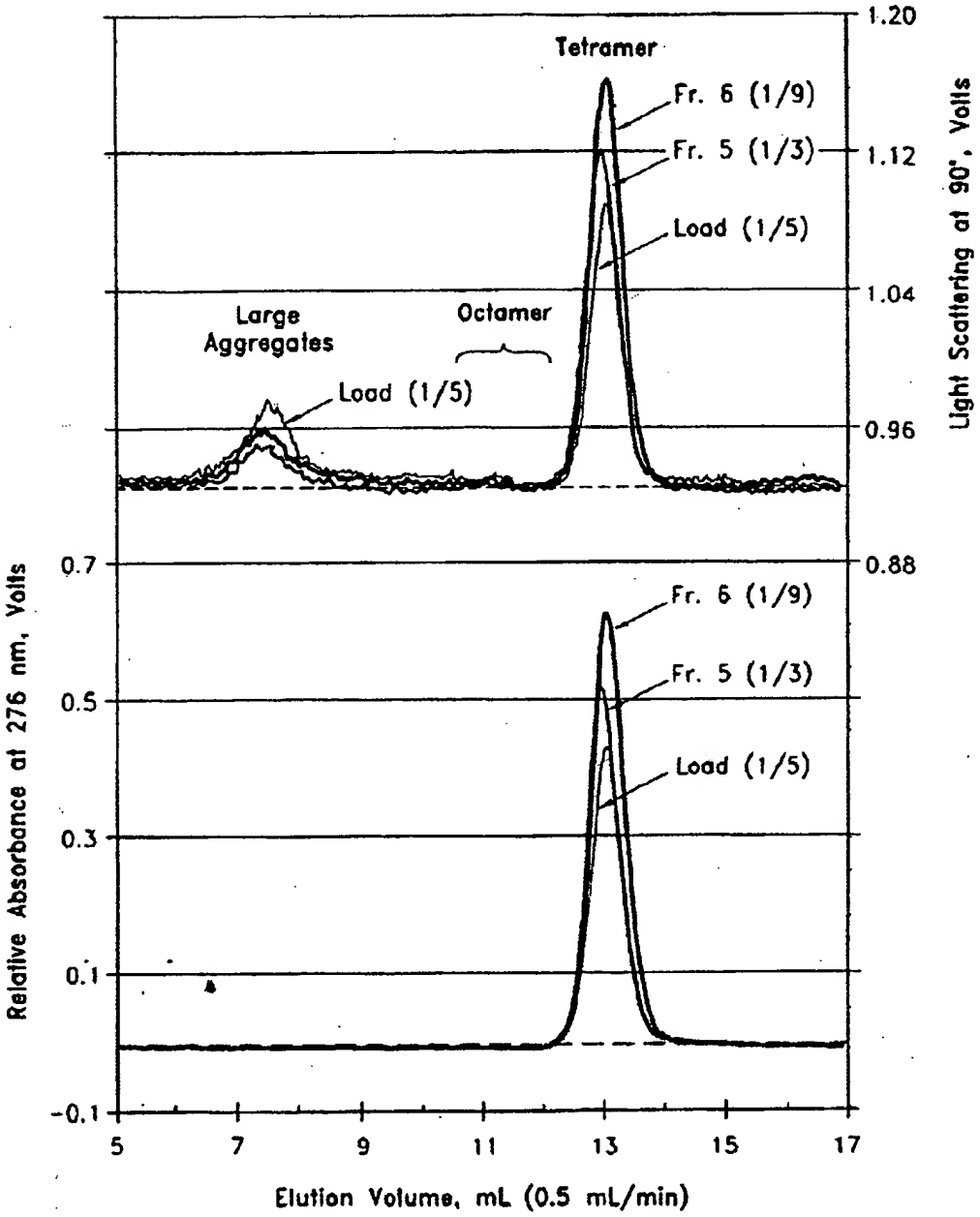

Figure 2
U.S. Appl.No 09/839,946